Quantitation and Localization of Matrix Metalloproteinases and Their Inhibitors in Human Carotid Endarterectomy Tissues

Salman Choudhary, Catherine L. Higgins, Iou Yih Chen, Michael Reardon, Gerald Lawrie, G. Wesley Vick III, Christof Karmonik, David P. Via, Joel D. Morrisett

Background—Matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) play a central role in arterial wall remodeling, affecting stability of fibrous caps covering atherosclerotic plaques. The objective of this study was to determine the spatial distribution of TIMP mass and MMP mass and activity of carotid endarterectomy (CEA) tissues and relate it to the distribution of atherosclerotic lesions.

Methods and Results—Fresh CEA tissues were imaged by multicontrast MRI to generate 3D reconstructions. Tissue segments were cut transversely from the common, bifurcation, internal, and external regions. Segments were subjected to total protein extractions and analyzed by ELISA for MMP-2 and -9 and TIMP-1 and -2 mass and by zymography for gelatinase activity. Segments at or near the bifurcation with highly calcified lesions contained higher MMP levels and activity than segments distant from the bifurcation; highly fibrotic or necrotic plaque contained lower MMP levels and activity and higher TIMP levels. Fatty streak, fibroatheroma with hemorrhage and calcification, and fully occluded lesions were enriched in MMP-2, MMP-9, and TIMP-1 and TIMP-2, respectively.

Conclusion—The spatial distribution of MMPs and TIMPs in carotid atherosclerotic lesions is highly heterogeneous, reflecting lesion location, size, and composition. This study provides the first semi-quantitative maps of differential distribution of MMPs and TIMPs over atherosclerotic plaques. (Arterioscler Thromb Vasc Biol. 2006;26:2351-2358.)

Key Words: carotid artery ■ atherosclerosis ■ MMPs ■ TIMPs ■ MRI

Advanced atherosclerotic plaques typically have a lipid-rich core covered by a fibrous cap composed of smooth muscle cells (SMC) and extracellular matrix (ECM). Plaque vulnerability is influenced by overall size, core size, cap thickness, cap inflammation, and cap fatigue. Fibrous caps are composed mainly of collagen, which determines cap tensile strength. Matrix composition affects several events in lesion development, including cell migration and proliferation, lipoprotein retention, cell adhesion, calcification, thrombosis, and apoptosis.

Degradation of ECM, which may weaken the fibrous cap resulting in plaque rupture, can be accomplished by macrophages through phagocytosis or secreted proteolytic enzymes, such as matrix metalloproteinases (MMPs). MMPs are a family of Zn$^{2+}$- and Ca$^{2+}$-dependent endopeptidases that degrade ECM proteins, such as gelatin, collagen, elastin, and fibrin, and play a central role in arterial wall remodeling. Secreted as zymogens (pro-MMPs) that must be activated by other proteases or reaction with organic mercurials, MMPs are active at neutral pH and can be inhibited by proteins including tissue inhibitors of metalloproteinases (TIMPs), by $\alpha_2$-macroglobulin, and by metal chelators such as phenanthrolines and EDTA. Although the MMP family consists of almost 20 known proteins, the present study has focused on MMP-2 and -9 and their role in carotid atherosclerosis.

MMP-2 (gelatinase A) is secreted as a 72-kDa proenzyme and primarily expressed in mesenchymal cells during development and tissue regeneration. Cleaving the N-terminal prodomain can be initiated by membrane-type MMPs or serine proteases. MMP-2 can degrade collagens, elastin, and fibronectin. With MMP-9, it degrades type IV collagen, the major component of basement membranes and gelatin.

MMP-9 (gelatinase B) has been identified in a number of cell types and has a broad range of specificities for native collagens, as well as gelatin, proteoglycans, and elastin. Secreted in a precursor form (pro–MMP-9, 92 kDa) that can be activated by MMP-3 (stromelysin-1) or bacterial proteinases, MMP-9 has been implicated in processes characteristic of inflammatory cells, uterine invasion of trophoblasts, and bone absorption and is thought to act synergistically with...
MMP-1 in degradation of fibrillar collagens as it degrades their denatured gelatin forms.\textsuperscript{9,10} Expressed by almost all activated macrophages, MMP-9 is the most prevalent form of MMP.

The natural plasma inhibitors of MMPs are TIMPs, which act to suppress matrix degradation and can be synthesized by monocytes/macrophages, SMC, and endothelial cells. Tissue activity requires a balance between MMP activation and TIMP inhibition, which is important in tissue remodeling, inflammation, tumor growth, and metastasis. TIMPs form 1:1 noncovalent complexes with MMPs and block access of substrates to catalytic sites. Four members of the TIMP family are known, 2 of which, TIMP-1 and -2, are part of this study focus.

Dysregulation of MMP/TIMP balance is a characteristic of extensive tissue degradation in certain degenerative diseases. Secretion of proteases at focal sites in plaques ultimately results in plaque instability and rupture. Therefore, localizing MMPs and TIMPs in atherosclerotic lesions is necessary for understanding the disease progression and regression.

MRI has become a powerful technology for imaging carotid atherosclerotic lesions in vivo. MRI was used to demonstrate that lipid-lowering with simvastatin is associated with significant regression of human carotid atherosclerotic lesions\textsuperscript{11} and to show that substantial low-density lipoprotein cholesterol reduction with rosuvastatin resulted in regression of lipid-rich necrotic core.\textsuperscript{12} Takaya et al\textsuperscript{13} demonstrated by MRI that hemorrhage into plaque accelerated progression. These and earlier studies\textsuperscript{14} attest to the value of MRI as a noninvasive method for accurately monitoring plaque dimensions (total vessel volume, normal wall volume, plaque volume, lumen volume) and composition (calcification, lipid, fibrous, thrombus).

### Materials and Methods

#### Tissue Acquisition and Storage

Carotid endarterectomy (CEA) specimens were obtained within 1 hour after surgical resection, digitally photographed, and stored until use in 50% glycerol/PBS (20°C) to preserve tissue morphology. Larger specimens having intact common, internal, and external branches were preferred for study (approved by an institutional review committee of Baylor College of Medicine; subjects gave informed consent).

#### Magnetic Resonance Imaging

Tissues were washed in PBS and transferred to a specially fabricated sample holder, permitting simultaneous imaging of 4 samples.\textsuperscript{15} The holder oriented the tissue long axis along the y-axis of the magnet so that 2-mm coronal slices gave axial images (supplemental Figure I, available online at http://atvb.ahajournals.org). A General Electric XL Enhance system operating at 1.5 T equipped with 6-cm phased array coils (Pathway Biomedical, Redmond, Wash) was used to acquire proton density weighted (PD-W), T1 weighted (T1W), and T2 weighted (T2W) images under conditions similar to those described previously.\textsuperscript{16}

#### Tissue Segmentation and Digital Photography

CEA specimens were cut into 5-mm segments from the bifurcation into the common, external, and internal carotids. Microscopic images of segments were acquired using a Leica DC300 digital camera attached to a Stereomaster dissecting microscope to document features frequently lost during processing (eg, thrombus, calcification) and to capture subtle textural and morphological features not always detected by other techniques. Using these images, lesion composition was evaluated and lesion categories were assigned.\textsuperscript{17}

### Protein Extraction

Tissues were extracted for total protein by the following methods.

#### Mild Conditions

Tissue segments (tissue nos. 958, 973, 974, 991, 1006) were transferred to tared 15-mL plastic culture tubes and weighed. Segments were then incubated with 2 mL of DMEM containing 4 µL of gentamycin (50 µg/100 mL) while agitated gently at room temperature for 15 hours, after which the extract medium was removed and stored (−20°C) for analysis.

#### Moderate Conditions

Moderate conditions sequentially followed mild conditions. Tissue segments (tissue nos. 958, 973, 974, 991, 1006) were transferred to tared 15-mL plastic culture tubes and weighed. To each tube was added 3 mL of DMEM containing 6 µL of gentamycin (50 µg/100 mL) and 0.1% octyl glucoside. Tissues were homogenized (Brinkmann Polytron; 10-second bursts, 50% power) until segments were completely dispersed into homogeneous suspensions. Extensively calcified tissues required multiple homogenization bursts. Homogenates were centrifuged at 3000 rpm (30 minutes). Supernatants were decanted into cryovials and stored (−20°C) until analyzed.

#### Stringent Conditions

Tissues (tissue nos. 960, 968, 985, 989, 1000) were transferred to tared 15-mL plastic culture tubes and weighed. To each tube was added 3 mL of extraction buffer (50 mmol/L 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid [HEPES], 150 mmol/L NaCl, 1 mmol/L ethylene glycol-bis[2-aminoethylether]-N,N,N',N'-tetra-acetic acid [EGTA], 10 mmol/L sodium pyrophosphate, 100 mmol/L NaF, 1.5 mmol/L MgCl\textsubscript{2}, 10% glycerol, 1% Triton X-100 [pH 7.5]). Tissues were homogenized as described for moderate conditions.

### Measurement of MMPs and TIMPs in Tissue Extracts

Human total MMP-2,7,8 total MMP-9,6,9,10 TIMP-1,18,19 and TIMP-2\textsuperscript{20,21} were measured by enzyme-linked immunosorbent assay (ELISA) with reagents from R&D Systems (Minneapolis, Minn) following the instructions in the product insert. MMP and TIMP data were normalized for extraction efficiency (supplemental Table I) and total protein and were expressed in normalized form unless otherwise stated. Proteins extracted under mild and moderate conditions were analyzed separately, and the individual data were added together.

### Zymography

Zymography was performed on total protein extracts from CEA tissue segments. Zymograms were run on 10% gels containing gelatin (Bio-Rad, Richmond, Calif) using the procedure described in the package insert. After electrophoresis, gels were renatured by incubation in detergent-free buffer, then activated by equilibration in activation buffer (50 mmol/L Tris hydroxymethyl aminomethane [TRIS], 150 mmol/L NaCl, 10 mmol/L CaCl\textsubscript{2}, 1 mmol/L ZnCl\textsubscript{2}, 0.02% Na\textsubscript{2}EDTA, pH7.5; 37°C, 4 hours). Gels were stained with Coomassie blue (0.05%, 30 minutes). Areas in which gelatin was digested appeared as clear bands (supplemental Figure III). ImageJ software was used to quantify bands. Enzyme activity was expressed in terms of band area produced per nanogram of pure MMP-9 (Oncogene, Boston, Mass). A standard curve for each gel was generated from 0.25, 0.50, 1.00, and 2.00 ng of pure MMP-9. The area of each band was integrated individually and plotted on a histogram showing relative contribution to total gelatinase activity.

### Three-Dimensional Reconstructions and MMP Data Fusion

Three-dimensional renderings of CEA tissues were generated using MRI slices by first interpolating the image data to contiguous slices...
of 1-mm thickness as the lowest common denominator between the MRI slice thickness of 2 mm and the tissue segment thickness of 5 mm. The grayscale pixel values of the CEA tissue in each slice was then mapped to MMP enzyme activity in the following manner: MMP activity of 7 ng/mg total protein (as upper limit of MMP activity for all tissues) was assigned a grayscale value of 255. For each tissue segment (5 of the 1-mm slices), a grayscale value proportional to MMP activity was calculated by dividing the segment MMP activity by 7 and multiplying by 255. This modified image data was stored in raw image data format. All image manipulations were performed using the ImageJ software package developed by Wayne Rasband (Research Services Branch, National Institute of Mental Health, Bethesda, Md; http://rsb.info.nih.gov/ij). Next, the Paraview software package (http://www.paraview.org) was utilized to create 3D-surface rendered reconstructions with a color lookup table mapping the grayscale pixel value 0 to purple and the grayscale pixel value 255 to red.

### Statistical Analysis

Plots indicating extent of association among MMP activity in CEA segment extracts (as determined by gelatin zymography) and MMP and TIMP masses (as determined by ELISA) were generated. The association between different analytes was determined by Spearman correlation analysis. Correlations with \( P < 0.05 \) were considered statistically significant.

### Results

The PD-W magnetic resonance (MR) images show discrete 2-mm slices (supplemental Figure II) corresponding to tissue contained within specific 5-mm segments (supplemental Figures IV through XIII). These images (32 slices acquired, 4 shown) capture features seen in macroscopic images of intact tissues (Figure 1) and microscopic images of segments (supplemental Figures IV through XIII). For example in tissue 960, slices 14 of 32 and 18 of 32, corresponding to segments I1 and Bd (supplemental Figure II), exhibit large hypointense stenotic regions attributable to calcification as verified by coregistered digital photographs (supplemental Figure V). These 2D MR images were used to reconstruct 3D images that served as templates onto which segment properties (eg, enzyme activity, protein mass) were mapped.

The MR images, macroscopic pictures, and microscopic photos each indicated considerable structural and compositional heterogeneity. Frequently, the bifurcation segment(s) had the greatest lesion burden, diminishing in the common and internal carotid segments with increasing distance from the flow divider. Typically, the external carotid contained little if any lesion and appeared as a patent, whitish tube (Figure 1: no. 985, E1 through E3; no. 989, E1 through E3). The high calcification content of some CEA tissues made it difficult to obtain histological sections that retained all the original components. For this reason, we used digital photography to document segment composition. The value of this approach is illustrated in supplemental Figure V, which shows extensive calcification in segments C1, Bp, Bd, and I0 (no. 960). The 3D visualization of this component is difficult if not impossible to duplicate by conventional histology of paraffin or frozen sections.

The heterogeneous distribution of macroscopic components suggested heterogeneous distribution of molecular components, such as MMPs and TIMPs. Rather than perform qualitative immunohistochemical staining for these proteins in situ, we chose to extract them from the tissue segments and analyze them by ELISA. This approach enabled quantitation of not only the individual proteins but also composite enzymatic activity. Three different extraction procedures were used. Forty segments cut from 5 CEA tissues (nos. 958, 973, 974, 991, 1006) were subjected first to the mild then moderate extraction procedure, and 42 segments cut from 5 tissues (nos. 960, 968, 985, 989, 1000) were subjected to the stringent procedure.

The effect of each extraction buffer on the immunoreactivity of each MMP and TIMP was determined by comparing the immunoreactivity of each pure protein before and after its exposure to the extraction buffer. Mild, moderate, and stringent buffers led to immunoreactivity recoveries of 88 to 97, 92 to 107, and 73% to 99%, respectively, for each MMP and TIMP (supplemental Table I).

The effect of the extraction process on MMPs and TIMPs was determined by measuring the percent recovery of immunoreactivity of exogenous protein added to normal CEA tissues subjected to one cycle of extraction. The process allowed recovery of 61 to 100, 81 to 107, and 61% to 118% immunoreactivity under mild, moderate, and stringent conditions, respectively (supplemental Table I).

The total extraction efficiency for MMPs and TIMPs was evaluated by measuring the immunoreactivity of endogenous protein in CEA tissue subjected to three extraction cycles, then extrapolating to 0 cycles. This measurement compensated for the effect of buffer and the extraction process on the
total amount of MMPs and TIMPs measured by ELISA. The extraction efficiency for mild, moderate, and stringent buffers was 73 to 100, 69 to 100, and 68% to 100%, respectively (supplemental Table I).

The extraction efficiency values were used to correct the MMP and TIMP values measured by ELISA. Corrected values were plotted as positive histograms for MMP-2 and MMP-9 and as negative histograms for TIMP-1 and TIMP-2 (Figure 2). Each histogram represents the mass of these proteins as a function of tissue segment position.

Several of the tissues studied were highly calcified at or near the bifurcation (eg, supplemental Figure V; no. 960: I1, I0, Bd, Bp). These segments were rich in MMPs and poor in TIMPs (Figure 2). In other CEA tissues, the bifurcation was very fibrotic and/or necrotic (supplemental Figure XII; no. 1000: B1, B, I1, I2, I3). These segments were poorer in MMPs and richer in TIMPs, especially TIMP-1. In still other tissues, the bifurcation area contained a thrombus (supplemental Figure VI; no. 968: B, I1). These segments were also rich in MMPs, especially MMP-9 (Figure 2).

Although ELISA measurements of MMPs and TIMPs indicate absolute abundance of these proteins in tissue extracts, this information does not necessarily indicate net gelatinase activity operating on the tissue. This activity was determined by gelatin zymography. Major bands, with molecular masses of 100 and 88 kDa, corresponding to MMP-9, and with 70 and 62 kDa, corresponding to MMP-2 were observed (supplemental Figure III). An additional band with a molecular mass of 130 kDa, not previously assigned to an individual MMP, was observed in some but not all extracts. The combined MMP-9 (or MMP-2) band areas for each extract sample were taken as a quantitative measure of MMP-9 (or MMP-2) activity. The MMP-9 activity, expressed as band area per unit MMP-9 mass, was significantly associated with MMP-9 abundance in some tissues (eg, nos. 960, 968, 973, 989, 1006) but not in others (eg, nos. 958, 965, 985, 991, 1000). Similarly, MMP-2 mass was also correlated with activity in some tissues (eg, nos. 958, 991, 1006) but not in others (eg, nos. 960, 968, 974, 974, 985, 989, 1000). This variability of statistically significant association between MMP-2 or MMP-9 mass and gelatinase activity in individual tissues may be due in part to the variation in TIMP-1 and TIMP-2 abundance. However, when the data from all ten tissues were combined, the mass of MMP-9 in individual tissue segment extracts was correlated (r=0.61, P<0.0001, n=42) with the activity of MMP-9. Likewise, the mass of MMP-2 was correlated (r=0.43, P=0.004, n=42) with activity of MMP-2.
To enhance visualization of gelatinase activity distribution over the CEA tissues, total activity values (0 to 7 MMP-9 ng equivalents) were color-coded then mapped onto 3D reconstructions of tissue nos. 960, 968, 985, 989. These representations indicate that maximum activity is localized to diseased segments at or near the bifurcation including the common and internal but not the external carotid.

Discussion

CEA tissues are highly heterogeneous, containing some areas that are grossly normal and other areas that are extensively stenosed. By cutting the common, internal, and external carotid branches into multiple segments, we were able to isolate normal and diseased regions and characterize their morphology, dimensions, and composition (Figure 1).

The conventional approach for characterizing atherosclerotic plaque morphology has been histological analysis of paraffin or frozen sections. However, many CEA tissues are so structurally fragile that they are not amenable to this approach. For this reason, we elected to use digital photography to capture images that showed features often lost during sectioning and staining procedures.

Immunohistochemistry is extensively used for depicting distribution of specific proteins in tissues. However, the results are usually qualitative and do not readily allow reasonably accurate quantitation. Because we wanted to quantitatively compare several different protein components in the same tissue segment, we chose to perform total protein extraction on each segment, then measure analytes of choice in the extract. When accompanied by careful controls, the total extract approach enables measurement of selected protein mass and activity by ELISA and zymography, respectively. In a separate study, we have used these extracts for protein microarray analysis.

In the present study, we have used 2D MR image slices to reconstruct 3D images of the tissues. These 3D images serve as useful templates onto which specific molecular components (eg, protein, mRNA) can be quantitatively mapped, a capability not available from 2D digital photographs.

In this study, 10 CEA tissues were cut into ~80 segments that were characterized in terms of morphology, MMP and TIMP composition, and gelatinase activity. Segments at or adjacent to the bifurcation were significantly stenosed, containing varying amounts of lipid, thrombus, calcification, and/or fibrotic material. The accumulation of these plaque components is usually attended by arterial remodeling, leading to net formation of connective tissue and fibrous or lipid accumulation and were classified as types I to III. Most of the segments in the bifurcation and proximal internal carotid contained large fibroatheroma frequently with hemorrhage and/or calcification and hence were classified as type IVa. This lesion was the most abundant (36%) of all the 10 types, with IVb being the next most abundant (15%). Significantly, the type II lesions contained more MMP-2 than any other lesion type, whereas type IVc lesions contained the most MMP-9, suggesting that type IVc lesions are undergoing considerable remodeling (Figure 4). Lesion type Vd contained much greater amounts of TIMP-1 and TIMP-2 than any other lesion type. This finding suggests that type Vd lesions have depressed MMP activity and suppressed remodeling, leading to net formation of connective tissue and almost complete occlusion (supplemental Figure XII; no. 1000: B1, I1, I2).

Mapping total gelatinase activity on 3D CEA images reconstructed from 2D MRI slices provides a graphical method for efficiently conveying quantitative information (Figure 5). Tissue nos. 960 and 989 had the highest level of activity (~7 MMP-9 ng equivalents); this activity was concentrated in the highly lesioned bifurcation and adjacent...
segments. Tissues with lower levels of activity still exhibited their maximal amount at or near the bifurcation.

Intraplaque hemorrhage is common in advanced coronary atherosclerotic lesions. Significantly, hemorrhage has been shown to increase expression of MMPs, including MMP-2 and MMP-9, in lesions. In the present study, a number of carotid atheroma segments exhibited sites of intraplaque hemorrhage (eg, supplemental Figure V: no. 968, Bd, I1, I2; supplemental Figure X: no. 989, C2, C1, B). These segments contained considerable amounts of MMP-2 and MMP-9 (Figure 2). The accumulation of MMPs at these sites may be attributable in part to the infiltration of macrophages, cells known to secrete MMPs and destabilize plaques.

Figure 3. MMP activity reported as the activity displayed by 1 ng of pure MMP-9 and as determined by gelatin zymography for human CEA tissue segments (nos. 960, 968, 989, 1000). As many as 5 bands (62, 70, 88, 100, 130 kDa) were observed in some cases and all are displayed. Percentages of total MMP activity for each band are indicated.

Figure 4. Distribution of MMP-2, MMP-9, TIMP-1, and TIMP-2 among CEA segments characterized by the lesion classification system developed by the American Heart Association Committee on Vascular Lesions and Virmani et al. Type II lesions contained the most MMP-2. Type IVc lesions had the highest content of MMP-9. Type Vd lesions contained the most TIMP-1 and TIMP-2. Histogram height represents the mean level of a specific MMP found in a particular lesion type.
The increasing body of evidence indicating the correlation of MMP and TIMP tissue levels with plaque stability\(^3\) raises the question of how plasma levels of these proteins might be involved. This question has been addressed in a recent study by Sapienza et al\(^3\) of MMP-1, -2, and -9 and TIMP-1 and -2 levels in patients undergoing endarterectomy. The plaques from patients were classified histologically as stable or unstable and their plasma MMP levels measured before and after surgery. Plasma MMP levels were higher in patients with unstable plaques compared with patients with stable plaques. In contrast, plasma levels of TIMPs were lower in patients with unstable plaques compared with stable plaques. This observation indicates that plasma levels of MMPs and TIMPs may be important biomarkers for carotid plaque instability.

Numerous studies have demonstrated that MMP-2 and MMP-9 are essential for SMC behavior and hence play a major role in fibrous cap formation and healing. However, imbalance in tissue levels of MMPs and TIMPs may be a cause of plaque instability. This condition might be treated by decreasing the level or activity of the enzymes. A feasible approach has been suggested by Bellosta et al.,\(^3\) who have observed that 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors decrease MMP-9, an effect mediated by suppressing synthesis of mevalonate, a precursor of multiple intermediates essential for several cellular functions. Other approaches might include intervention with novel inhibitors of MMP gene expression or activity.

**Conclusion**

CEA tissue segments have been used to investigate the distribution and abundance of MMPs and TIMPs in atherosclerotic plaques. MMP-9 was highly abundant in calcified segments at or near the bifurcation. MMP-9 was very abundant in segments with intraplaque hemorrhage. Grossly normal segments contained lesser amounts of MMPs, which were predominantly MMP-2. TIMPs were lowly abundant in calcified plaques and more highly abundant in fibrotic and necrotic segments. This study provides the first semiquantitative maps of differential distributions of MMPs and TIMPs over an atherosclerotic plaque.

**Figure 5.** Three-dimensional reconstructions of CEA tissues (Figure 1) from multiple 2D MRI slices (supplemental Figure II; corresponding arrows) with fusion of MMP enzyme activity (Figure 3). Color-coded activity data were fused onto 3D renderings as described in the Materials and Methods. The maximal MMP activities from which the color levels were calibrated for tissue nos. 960, 968, 985, and 989 were 6.75, 3.12, 3.55, and 6.96 ng/mg total extracted protein, respectively.

**Acknowledgments**

We thank Seth Marvel for assistance with zymogram analysis.

**Sources of Funding**

This work has been supported in part by National Institutes of Health grants R01HL63090 and T32HL07812. (TTGA)

**Disclosures**

None.

**References**


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Arterioscler Thromb Vasc Biol. 2006;26:2351-2358; originally published online August 3, 2006; doi: 10.1161/01.ATV.0000239461.87113.0b

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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### Supplement Table 1

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1Percent recovery of immunoreactivity of purified MMPs and TIMPs after exposure to the extraction buffer. Values represent means of triplicate determinations.

2Percent recovery of immunoreactivity of exogenous MMPs and TIMPs added to CEA tissues subjected to one extraction cycle. Values represent means of triplicate determinations on 2-3 tissues.

3Levels of MMPs and TIMPs from three extraction cycles.

4Percent extrapolated immunoreactivity of endogenous MMPs and TIMPs in CEA tissues subjected to three extraction cycles. Values represent means of triplicate determinations on 2-3 tissues.

5Reciprocal of fraction extrapolated immunoreactivity used to correct ELISA results (Figure 4A).
### Supplement Table 2

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<th>Tissue #958</th>
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<td>C2: Type I</td>
<td>C3: Type III intermediate</td>
<td>C3: Type I</td>
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<td>C1: Type IVb plaque hemorrhage</td>
<td>C2: Type III intermediate</td>
<td>C2: Type I</td>
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<tr>
<td>B: Type IVb plaque hemorrhage</td>
<td>C1: Type IVa with calcified nodules</td>
<td>C1: Type II fatty streak</td>
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<td>I1: Type IVc calcified nodule + hemorrhage</td>
<td>Bp: Type IVb with calcified nodules</td>
<td>B: Type Ivc</td>
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<td>I2: Type IVb + hemorrhage</td>
<td>Bd: Type IVb with calcified nodules</td>
<td>I1: Type IVb + hemorrhage</td>
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<tr>
<td>I3: Type Va fibroatheroma</td>
<td>I1: Type IVc with calcified nodules</td>
<td>I2: Type IVb + hemorrhage</td>
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<tr>
<td>E: Type Vd</td>
<td>I2: Type Va fibroatheroma + hemorrhage/Ca2+</td>
<td>E1: Type II fatty streak</td>
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<td></td>
<td>I3: Type Va fibroatheroma + hemorrhage/Ca2+</td>
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<td>I4: Type III intermediate</td>
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<td>C2: Type IVa + hemorrhage</td>
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<tr>
<td>C2: Type IVa fibrocalcific</td>
<td>C3: Type IVb + hemorrhage/Ca2+</td>
<td>C1: Type IVa + hemorrhage</td>
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<td>C1: Type IVa fibrocalcific</td>
<td>C2: Type IVb + hemorrhage/Ca2+</td>
<td>Bp: Type IVa fibroatheroma + hemorrhage</td>
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<td>B1: Type IVa</td>
<td>C1: Type IVb</td>
<td>Bd: Type IVa fibroatheroma + hemorrhage</td>
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<td>B2: Type IVa</td>
<td>Bd: Type IVb + hemorrhage/Ca2+</td>
<td>I1: Type IVa fibroatheroma + hemorrhage</td>
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<tr>
<td>I1: Type IVb vulnerable plaque + old thrombus</td>
<td>I1: Type III intermediate</td>
<td>I2: Type IVa fibroatheroma + hemorrhage</td>
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<td>E1: Type IVa fibrolipid</td>
<td>E2: mild Type III</td>
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<td>C2: Type IVa + hemorrhage</td>
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<td>C2: Type III intermediate</td>
<td>C1: Type IVa + hemorrhage</td>
<td>C2: Type IVa + hemorrhage</td>
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<tr>
<td>C1: Type Va + calcified nodules</td>
<td>B: Type IVa + hemorrhage</td>
<td>C1: Type IVa + hemorrhage</td>
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<tr>
<td>B1: Type Va + calcified nodules</td>
<td>I1: Type IVa</td>
<td>B1: Type Vd</td>
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<td>I0: Type Va + calcified nodules</td>
<td>I2: Type IVa</td>
<td>B: Type IVc</td>
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<td>I1: Type Va + calcified nodules</td>
<td>E1: Type II fatty streak</td>
<td>I1: Type Vd</td>
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<tr>
<td>I2: Type Va + calcified nodules</td>
<td>E2: Type II</td>
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<td>I3: Type Va + calcified nodules</td>
<td>E3: Type I</td>
<td>I3: Type IVb + hemorrhage</td>
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<td>I4: Type Va + calcified nodules</td>
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<td>E1: Type IVa</td>
<td>C1: Type IVa fibroatheroma</td>
<td>I3: Type Vc old thrombus</td>
</tr>
<tr>
<td>E2: Type IVa</td>
<td>Bp: Type IVa atheroma</td>
<td>I4: Type Ivc + hemorrhage</td>
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<td>Bd: Type IVa (E) &amp; Vc (I)</td>
<td>E1: Type III fatty streak</td>
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<td>I1: Type Vc old thrombus</td>
<td>E2: Type III fatty streak</td>
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<tr>
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<td>I2: Type Vc old thrombus</td>
<td>E3: Type III fatty streak</td>
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(A) Multi-sample holder and tank used for acquiring MR images of CEA tissues *ex vivo* under conditions resembling *in vivo*. (B) RF coils (6 cm, phased array) fitted around the tank.
Supplement Figure 2

Four selected 1.5T PDW MR images of CEA tissues #960,968,985,989. The selected slices (2mm) fall within the indicated segments (5mm) cut from the tissues. Images were acquired under conditions similar to those used for in vivo imaging.
Zymogram of total protein extracts from segments C3,C1,C2,B,B2 cut from a CEA specimen. A standard curve for the gel was generated from 0.25, 0.50, 1.00, and 2.00ng of pure MMP-9. The area of each band was integrated individually and plotted on a histogram showing its relative contribution to the total gelatinase activity.
Supplement Figure 4A

958-C2
Type I

958-C1/B
Type IVb plaque hemorrhage

958-I1/B
Type IVb plaque hemorrhage

958-I1p
Type IVc Calcified nodule + hemorrhage

958-I1d
Type IVc Calcified nodule + hemorrhage

958-I2
Type IVb + hemorrhage

958-I3
Type IVa fibroatheroma

958-E/B
Type Vd

958-E1
Type Vd
Supplement Figure 4B

Level of MMPs and Inhibitors (ng/mg of protein)

Tissue segments

C2  C1  I1  I2  I3  E1

Mean

67%  33%  42%  58%  47%  53%  30%  70%  57%  43%  30%  67%  70%  43%  55%  57%  33%  47%  75%  33%  47%  55%  45%
Type III intermediate

Type IVa fibroatheroma + hemorrhage/Ca²⁺

Type IVc with calcified nodules

Type IVb with calcified nodules

Type IVc with calcified nodules

Type IVa fibroatheroma + hemorrhage

Type III intermediate
Supplement Figure 6

**Type I**

- **968-C3**
  - Type I

- **968-C2**
  - Type I

- **968-C1**
  - Type II fatty streak

**Type IVc**

- **968-Bd**
  - Type IVc

**Type IVb + hemorrhage**

- **968-I1**
  - Type IVb + hemorrhage

- **968-I2**
  - Type IVb + hemorrhage

- **968-E1**
  - Type II fatty streak
Supplement Figure 7A

973-C3  
Type I

973-C2  
Type IVa fibrocalcific

973-C1  
Type IVa fibrocalcific

973-B1p  
Type IVa

973-B1d  
Type IVa

973-B2  
Type IVa

973-I1  
Type IVb vulnerable plaque
+ old thrombus

973-I2  
Type IVa + hemorrhage
+ old thrombus

973-I3  
Type IVa + hemorrhage

973-I4  
Type II

973-E1  
Type IVa fibrolipid
Supplement Figure 8B

Level of MMPs and Inhibitors (ng/mg of protein)

Tissue segments

- C4 - C3 - C2 - C1 - B - I1 - I2 - E1

Mean flows:
- MMP 2: 78%
- MMP 9: 22%

Bar heights represent levels, with percentages indicating the mean values for each segment.

- C4: 96% (MMP 2), 5% (MMP 9)
- C3: 97% (MMP 2), 3% (MMP 9)
- C2: 99% (MMP 2), 1% (MMP 9)
- C1: 99% (MMP 2), 4% (MMP 9)
- B: 73% (MMP 2), 27% (MMP 9)
- I1: 66% (MMP 2), 44% (MMP 9)
- I2: 44% (MMP 2), 56% (MMP 9)
- E1: 56% (MMP 2), 45% (MMP 9)

Bar colors and patterns indicate different inhibitors:
- TIMP 1: Blue
- TIMP 2: Magenta
- MMP 2: Red
- MMP 9: Green

Note: The graph shows the level of MMPs and inhibitors in different tissue segments, with mean values indicated for MMP 2 and MMP 9. The bars represent the percentage levels, with colors and patterns used to distinguish between MMPs and inhibitors.
Supplement Figure 9A

985-C2
Type IVa + hemorrhage

985-C1
Type IVa + hemorrhage

985-Bp
Type IVa fibroatheroma + hemorrhage

985-Bd
Type IVa fibroatheroma + hemorrhage

985-E1
Mild Type III

985-E2
Mild Type III

985-E3
Mild Type III

985-I1
Type IVa fibroatheroma + hemorrhage

985-I2
Type IVa fibroatheroma + hemorrhage

985-I3
Type IVa fibroatheroma + hemorrhage

985-I4
Type IVa fibroatheroma + hemorrhage
Supplement Figure 10

989-C2
Type IVa + hemorrhage

989-C1
Type IVa + hemorrhage

989-C1e
Type IVa + hemorrhage

989-B
Type IVa + hemorrhage

989-I1
Type IVa

989-I2
Type IVa

989-E1
Type II fatty streak

989-E2
Type II

989-E3
Type I
Supplement Figure 11A

- **991-C3** Type III
- **991-C2** Type III intermediate
- **991-C1/B** Type Va + calcified nodules
- **991-I0/B1** Type Va + calcified nodules
- **991-I0** Type Va + calcified nodules
- **991-I1** Type Va + calcified nodules
- **991-I2** Type Va + calcified nodules
- **991-I3** Type Va + calcified nodules
- **991-I4** Type Va + calcified nodules
- **991-E/B** Type IVa
- **991-E1p** Type IVa
- **991-E2** Type IVa
Supplement Figure 12

1000-C3p  Type IVa + hemorrhage
1000-C2   Type IVa + hemorrhage
1000-C1d  Type IVa + hemorrhage
1000-B/IId Type IVc
1000-B/Ilp Type Vd
1000-I1p  Type Vd
1000-I2p  Type Vd
1000-I2d  Type Vd
1000-I3p  Type IVb
1000-I3d  Type IVb + hemorrhage
1000-B/Ed Type IVa + hemorrhage
Supplement Figure 13A

**1006-C1p**
Type IVa fibroatheroma

**1006-C1d**
Type IVa fibroatheroma

**1006-Bp**
Type IVa fibroatheroma

**1006-Bd**
Type IVa (E) & Type Vc (I)

**1006-I1**
Type Vc Old thrombus

**1006-I2**
Type Vc Old thrombus

**1006-I3**
Type Vc Old thrombus

**1006-I4**
Type IVc + hemorrhage

**1006-E1**
Type III fatty streak

**1006-E2**
Type III fatty streak

**1006-E3**
Type III fatty streak
Supplement Figure 13B

Tissue segments

Level of MMPs and Inhibitors (ng/mg of protein)

* insufficient sample

MMP 2

MMP 9

mean

63%

37%

mean

46%

46%